



PATENTS

Attorney Docket No. 021396-000200 (24011-0002)

**CERTIFICATE OF MAILING**

I hereby certify that this paper is being deposited with the U.S. Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, DC 20231, on 6/25/02

*[Signature]*

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of

Ivarie et al.

Application No: 09/173,864

Filed: October 16, 1998

Title: NOVEL TRANSGENIC BIRDS AND THEIR EGGS

Assistant Commissioner for Patents  
Washington, DC 20231

Sir:

**RECEIVED**  
JUL 05 2002  
TECH CENTER 1600/2900

: Group Art Unit: 1636  
: Examiner: Sumesh Kaushal

**COPY OF PAPERS  
ORIGINALLY FILED**

**DECLARATION UNDER 37 CFR 1.132**

I, Jeffrey Rapp, Ph.D., hereby declare as follows:

1. I am a staff scientist employed by AviGenics, inc., of Athens, GA., licensee of the subject application, since June 1999. I consider myself to be skilled in the art of avian transgenesis. My curriculum vitae is appended hereto.

2. The following experiments were conducted by me personally, or by those working under my supervision, to produce a transgenic chicken and egg expressing an exogenous protein, following the procedures set forth in the subject application of Ivarie, *et al.*

3. These experiments were designed to demonstrate the expression of human erythropoietin (EPO) in a chicken and egg, as suggested at page 32, line 10 of the specification.

4. Following the teachings of Example 1 (Vector Construction) of the specification, an pNLB-MDOT-EPO vector was created, substituting an EPO encoding sequence for the BL encoding sequence of the Example. Instead of using the CMV promoter as shown in Example 1 we used a synthetic promoter (MDOT) having both ovomucoid and conalbumin (page 9, lines 17-19) sequence elements (pNLB-MDOT-EPO vector, a.k.a. pAVIJCR-A145.27.2.2).

The DNA sequence for human EPO based on hen oviduct optimized codon usage was created using the BACKTRANSLATE program of the Wisconsin Package, version 9.1 (Genetics Computer Group, Inc., Madison, WI) with a codon usage table compiled from the chicken (*Gallus gallus*) ovalbumin, lysozyme, ovomucoid, and ovotransferrin proteins. The DNA sequence was synthesized and cloned into the 3' overhang T's of pCRII-TOPO (Invitrogen) by Integrated DNA Technologies, Coralville, IA, on a contractual basis (Fig. 1). The EPO coding sequence was then removed from pEpoMM with *Hind* III and *Fse* I, purified from a 0.8% agarose-TAE Gel, and ligated to *Hind* III and *Fse* I digested, alkaline phosphatase-treated pCMV-IFNMM. The resulting plasmid was pAVIJCR-A137.43.2.2 which contained the EPO coding sequence controlled by the cytomegalovirus immediate early promoter/enhancer and SV40 polyA site. The plasmid pAVIJCR-A137.43.2.2 was digested with *Nco* I and *Fse* I and the appropriate fragment ligated to an *Nco* I and *Fse* I-digested fragment of pMDOTIFN to obtain pAVIJCR-A137.87.2.1 which contained EPO driven by the MDOT promoter. In order to clone the EPO coding sequence controlled by the MDOT promoter into the NLB retroviral plasmid, the plasmids pALVMDOTIFN and pAVIJCR-A137.87.2.1 were digested with *Kpn* I and *Fse* I. Appropriate DNA fragments were purified on a 0.8% agarose-TAE gel, then ligated and

transformed into DH5 $\alpha$  cells. The resulting plasmid was pNLB-MDOT-EPO (a.k.a. pAVIJCR-A145.27.2.2).

5. Following the procedures of Example 2 (Production of Transduction Particles), transduction particles of pNLB-MDOT-EPO were produced.

Senta packaging cells (Cosset *et al.*, 1990) were plated at a density of  $3 \times 10^5$  cells/35mm tissue culture dish in F-10 medium (Life Technologies) supplemented with 50% calf serum (Atlanta Biologicals), 1% chicken serum (Life Technologies), 50  $\mu$ g/ml hygromycin (Sigma), and 50  $\mu$ g/ml phleomycin (CAYLA, Toulouse, France). These cells were transfected 24 hours (h) after plating with 2  $\mu$ g of CsCl-purified pNLB-MDOT-EPO DNA and 5  $\mu$ l of Lipofectin liposomes (Life Technologies) in a final volume of 800  $\mu$ l Optimem (Life Technologies). The next day, medium from transfected Sentas was recovered and filtered through a 0.45 micron filter. This medium was then used to transduce Isolde cells. 0.5 ml of the filtered medium recovered from Senta cells was added to 6 ml of F-10 (Life Technologies) supplemented as described above, in addition to polybrene (Sigma) at a final concentration of 4  $\mu$ g/ml. This mixture was added to  $10^6$  Isolde packaging cells (Cosset *et al.*, 1990) plated on a 100mm dish the previous day, then replaced with fresh medium (as described for Senta growth) 4 h later. The next day, the medium was replaced with fresh medium which also contained 200  $\mu$ g/ml neomycin (G418, Sigma). Every other day, the medium was replaced with fresh F-10 medium containing the supplements described above for Senta growth, and also 200  $\mu$ g/ml neomycin. Seven to ten days later, single colonies were visible by eye and these were picked and placed into 24 well dishes. When the 24 well dishes became confluent, medium was harvested and titered to determine the cell lines with the highest production of retrovirus.

The Isolde cell line producing the highest titer of EPO-encoding transducing particles was scaled up to six T-75 tissue culture flasks. When flasks were confluent, cells were washed with F-10 medium (unsupplemented) and transducing particles were then harvested for 16 h in 14 ml/flask of F-10 containing 1% calf serum (Atlanta Biologicals) and 0.2% chicken serum (Life Technologies). Medium was harvested, filtered through a 0.45 micron syringe filter, then centrifuged at 195,000xg in a Beckman 60Ti rotor for 35 minutes. Liquid was removed except for 1 ml, and this was incubated with the pellet at 37°C with gentle shaking for one hour. Aliquots were frozen at -70°C. Transducing particles were then titered on Senta cells to determine concentrations used to inject embryos.

6. Following the procedures of Example 3 (Production of Transgenic Chickens), chimeric birds were produced.

Approximately 300 White Leghorn eggs were windowed according to the Speksnijder procedure (U.S. Patent No. 5,897,998), then injected with  $\sim 7 \times 10^4$  transducing particles per egg. Eggs hatched 21 days after injection, and human EPO levels were measured by EPO ELISA from serum samples collected from chicks one week after hatch.

7. Following the procedures of Example 10 (Production of Fully Transgenic G1 Chickens), males were selected for breeding.

In order to screen for G<sub>0</sub> roosters which contained the EPO transgene in their sperm, DNA was extracted from rooster sperm samples by Chelex-100 extraction (Walsh *et al.*, 1991). DNA samples were then subjected to Taqman™ analysis on a 7700 Sequence Detector (Perkin Elmer) using the "neo for-1" (5'-TGGATTGCACGCAGGTTCT-3') and "neo rev-1" (5'-GTGCCCAGTCATAGCCGAAT-

3') primers and FAM labeled NEO-PROBE1 (5'-CCTCTCCACCCAAGCGGCCG-3') to detect the transgene. Eight G<sub>0</sub> roosters with the highest levels of the transgene in their sperm samples were bred to nontransgenic SPAFAS (White Leghorn) hens by artificial insemination. Blood DNA samples were screened for the presence of the transgene by Taqman™ analysis as described above.

Out of 1,054 offspring, 16 chicks were found to be transgenic (G<sub>1</sub> birds). Chick serum was tested for the presence of human EPO by EPO ELISA, and EPO was present at ~70 nanograms/ml. Egg white in eggs from G<sub>1</sub> hens was also tested for the presence of human EPO by EPO ELISA and found to contain human EPO at ~70 nanograms/ml. The EPO present in eggs was biologically active when tested on a human EPO responsive cell line (HCD57 murine erythroid cells).

This declaration is made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. §1001) and may jeopardize the validity of the application or any patent issuing thereon. All statements made of my own knowledge are true and all statements made on information and belief are believed to be true.

June 3, 2002

Date

Jeffrey Rapp

Jeffrey Rapp, Ph.D.

---

Figure 1. Coding sequence of synthetic human EPO gene

ATGGGCGTGACGAGTGCCCTGCTTGGCTGTGGCTGCTCTTGAGCCTGCTCAGCCTG  
CCTCTGGGCCTGCCTGTGCTGGGCGCTCCTCCAAGGCTGATCTGCGATAGCAGGGT  
GCTGGAGAGGTACCTGCTGGAGGCTAAGGAGGCTGAGAACATCACCACCGGCTGC

Amendment

Attorney Document No. 021396-000200 (24011-0002)

Application No. 09/173,864

GCTGAGCACTGCAGCCTGAACGAGAACATCACCGTGCCTGATACCAAGGTGAACTT  
TTACGCTTGGAAGAGGATGGAGGTGGGCCAGCAGGCTGTGGAGGTGTGGCAGGGC  
CTGGCTCTGCTGAGCGAGGCTGTGCTGAGGGGCCAGGCTCTGCTGGTGAACAGCTC  
TCAGCCTTGGGAGCCTCTGCAGCTGCACGTGGATAAGGCTGTGAGCGGCCTGAGAA  
GCCTGACCACCCTGCTGAGGGCTCTGGGCGCTCAGAAGGAGGCTATCAGCCCTCCA  
GATGCTGCAAGCGCTGCCCCTCTGAGGACCATCACCGCTGATACCTTTAGGAAGCT  
GTTTAGGGTGTACAGCAACTTTCTGAGGGGCAAGCTGAAGCTGTACACCGGCGAGG  
CTTGCAGGACCGGCGATAGG